DESCRIPTION

NEURONAL CELL DEATH RECEPTOR

Technical Field

The present invention belongs to the field of "neurophysiology," specifically relates to neuronal cell death.

Background Art

Alzheimer's disease (AD) is one of neurodegenerative diseases on which histopathological research task is now most energetically pushed forward. The disease is generally characterized by progressive loss of neuronal cells, intraneuronal tangles, and extracellular senile plaques. The major constituent of the senile plaques is amyloid β protein (Aβ). Aβ is produced by being cleaved off from a transmembrane precursor called APP. Since APP_{695} having 695 amino acid residues was identified in 1987 (J. Kang et al., 1987, Nature, 325: 733), at least 10 types of APP isoforms are known to be produced from a single gene by alternative splicing (R. Sandbrink et al., 1994, J. Biol. Chem., 269: 1510). An APP_{695} which lacks protease-inhibiting region is expressed chiefly in the brain (R.L. Neve et al., 1988, Neuron, 1: 669; P. Ponte et al., 1991, Mol. Brain Res., 9: 259). An APP-deficient mouse has maldevelopment in the central nervous system (CNS) (U. Muller et al., 1994, Cell, 79: 755; H. Zheng et al., 1995, Cell, 81: 525). This finding suggests that APP has a physiological function in addition to the pathological function as the precursor of Aβ. According to a recent study using doubly-targeted mouse that is deficient in both APP and APLP2, which is an APP-like molecule exhibiting high homology to APP (C.S. von Koch et al., 1997, Neurobiol. Aging, 18: 661), APP is suggested to function in embryogenesis and in early neuronal development.

However, so far, there are only limited findings on the original function of APP. APP has been believed to participate in the proliferation of neuronal cells and axonal outgrowth (D. Schubert et al., 1989, Biochem. Biophys. Res. Commun. 162: 83; E.A. Milward et al., Neuron, 1992, 9: 129; M. P. Mattson et al., 1993, Trends Neurocsi.

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16: 409; D.H. Small et al., 1994, J. Neurosci. 14: 2117). However, these functions are associated with the soluble APP. What is known about membrane-bound APP is only the fact that membrane-bound APP is abundantly localized in the synapse. This suggests that transmembrane APP is involved in physiological function in the neuron (W. Schubert et al., 1991, Brain Res. 563: 184). The overexpression of APP in fibroblast cells inhibits their cellular adhesion. Therefore, APP is also recognized to function on cell surface (K. Ueda et al., 1989, Annals Neurol. 25: 246). Recently, APP has been believed to function as a cell surface receptor in neurons (A. Ferreira et al., 1993, J. Neurosci. 13: 3112; J.G. Culvenor et al., 1995, Exp. Cell. Res. 220: 474), based on recent analytical studies particularly on cell surface expression of APP in neurons (S.S. Jung et al., 1996, J. Neurosci. Res. 46: 336), specific interaction between APP and trimeric G protein Go (Nishimoto et al., 1993, Nature, 362: 75-79; Brouillet et al., 1999, J. Neurosci, 19: 1717-1727), interaction with Fe65, which is an intracellular Shc-like signal transduction factor (F. Fiore et al., 1995, J. Biol. Chem. 270: 30853), and others. *In vitro* experiments have also revealed that transmembrane APP_{695} has a function associated with signal transduction, that this function is regulated upon ligand binding (T. Okamoto et al., 1995, J. Biol. Chem. 270: 4205), and that the function associated with signal transduction is activated with each of three types of APP_{695} mutants found in patients with familial Alzheimer's disease (FAD) (T. Okamoto et al., 1996, EMBO J. 15: 3769). It has also been reported that overexpression of wild-type APP by using a virus vector results in degeneration in primary cultured neurons (I. Nishimura et al., 1998, J. Neurosci. 18: 2387).

It has been known that the transient introduction of APP gene carrying a familial AD-type mutation (FAD-APP) into F11 cells induces apoptosis of the cells (T. Yamatsuji et al., 1996, Science 272: 1349-1352). However, the gene was expressed and induced the death in only a small number of cells in this system, and the frequency of induced cell death strongly depending on the efficiency of gene introduction. Because of these, the previous system is unsuitable for the screening of anti-Alzheimer's disease eradicative drug that

antagonizes neuronal loss. The difficulty can be overcome by establishing a neuronal cell line that stably expresses FAD-APP, however, such attempts were entirely unsuccessful until now. It has also been unknown whether or not transmembrane APP_{695} functions as a receptor for neuronal cell death.

Disclosure of the Invention

An objective of the present invention is to provide a method for inducing neuronal cell death associated with APP activation. Another objective of the present invention is to provide an inducing agent for neuronal cell death associated with APP activation, an inhibitor or enhancer for the induction, a method for the screening of the inhibitor or enhancer, and a kit for the screening.

The present inventors deduced that the APP mutant was an active molecule that gave the constitutive signal; that, hence, the difficulty in the establishment of neuronal cell line that stably expresses FAD-APP could result from cell death of FAD-APP-expressing neuronal cell; and that, thus, the establishment of the cell line failed. Then, they considered that, on the contrary, threshold value of the intracellular signal input level for cell death, which depended on the expression level of normal APP, would be high enough to afford the cell line stably expressing APP. If it is possible to develop a system where cell death is inducible with nearly 100% efficiency by treating neuronal cells stably expressing APP with an antibody or the like functioning as an APP agonist, such a system is ideal for the screening of the antagonistic agent and the search for APP agonist, in which system the death can be induced in nearly all the cells and the cell mortality rate is constant. The previously possible system using FAD-APP serves as a neuronal cell death model for familial Alzheimer's disease, which comprises only a small rate among cases of Alzheimer's disease. If a system using normal APP is developed, it can be assumed that such a system serves as an *in vitro* model not for familial Alzheimer's disease, comprising only the minority of the disease, but for sporadic Alzheimer's disease, that comprises by far the majority of the disease, because neuronal cell death is induced by non-mutant APP in the system. The search for the antagonistic agent, which is conducted based on

this system, will greatly contribute to the development of agent for treating general Alzheimer's disease.

Then the present inventors constructed a vector for the expression of APP_{695}, which is a neuronal isoform of APP and lacks the protease-inhibiting region but retains the remaining APP regions including the extracellular region and intracellular region. The vector was introduced into a neuronal cell line F11 to provide F11 neuronal cells stably expressing APP_{695} (F11/APP cell). The cell was found to overexpress APP_{695} at about 20 times higher level relative to the parental F11 cell line. When the cells were treated with an anti-APP monoclonal antibody that recognized the extracellular domain of APP, it was found that DNA fragmentation and nuclear morphology changes characteristic of apoptosis were induced in nearly all the cells within 24 hours and the death occurred in almost all the cells in 72 hours. The inventors also investigated characteristics of the cell death induced by the anti-APP monoclonal antibody and APP and found chromosomal DNA laddering and nuclear compaction characteristic of apoptosis, as well as found that the cell death was inhibited by an inhibitor for caspase that is recognized to participate in apoptotic cell death. Further the inventors clarified that the anti-APP monoclonal antibody did not induce the death of glioma cells overexpressing APP_{695}. The fact that the anti-APP antibody directly interacts with APP on the neuronal cell surface and the ligand-specific APP activation induces the death of neuronal cell suggests that membrane-bound APP has a biological function as a receptor associated with cell death. From these findings, the present inventors have clarified that, depending upon APP agonist, APP induces neuron-specific apoptosis, which results in the death of neuronal cell.

The inventors further developed a system capable of inducing the expression of not normal APP but FAD-APP, which is an active form mutant of APP_{695} found in familial Alzheimer's disease. As described above, the previously developed cell lines expressing FAD-APP were transient expression systems, their FAD-APP expression efficiency is low and variable, and therefore the system is unsuitable for detailed analyses for cell death and screening of cell death inhibitors and

such. If it is possible to develop a cell system where the active APP mutant is hardly or not expressed without stimulation but the expression is induced in all the cells in the presence of an external stimulus, then the problems can be overcome. Hence, the inventors introduced a DNA encoding the active APP mutant into F11 cells, thereby achieving the construction of a new cell line in which the expression could be induced in response to ecdysone. The effect of ecdysone was tested by acting it on this system and, as a result, the expression of active APP mutant was induced. Thus it was demonstrated that the death could be induced in almost all the cells within a certain period of time. Like the death of F11/APP cell, the cell death event showed the characteristics of apoptosis. The fact also supports that the activation of membrane-bound APP is a cause of Alzheimer's disease.

The above-mentioned death-inducing system of neuronal cell can be used for the screening of APP antagonist and APP agonist. Compounds isolated by this screening are thought to be applied to prevention and treatment of neuronal disorders.

The present invention relates to a method for inducing neuronal cell death associated with APP activation, an inducing agent for neuronal cell death associated with APP activation, an inhibitor or enhancer for the induction, a method for the screening the inhibitor or enhancer, and a kit for the screening and more specifically relates to:

(1) a neuronal cell expressing a foreign APP that induces the death of the cell can be induced when activated;

(2) the neuronal cell of (1), wherein the APP is APP_{695};

(3) the neuronal cell of (1) or (2), wherein the cell is an F11 cell;

(4) the neuronal cell of (3), wherein the total intracellular expression level of endogenous APP and the foreign APP is about 20 times as much as the expression level of the endogenous APP alone;

(5) a neuronal cell inducibly expressing an active APP mutant that can induce , the death of the cell;

(6) the neuronal cell of (5), wherein the cell contains a vector comprising a DNA encoding a nuclear receptor and a vector comprising a DNA encoding an active APP mutant that is functionally connected

downstream of a responsive element for the nuclear receptor and wherein the expression of the active APP mutant can be induced by contact of a ligand with the nuclear receptor;

(7) the neuronal cell of (6), wherein the nuclear receptor is an ecdysone receptor and wherein the expression of the active APP mutant is induced by the treatment with ecdysone;

(8) the neuronal cell of any one of (5) to (7), wherein the cell is an F11 cell;

(9) a method for inducing neuronal cell death associated with APP activation, the method comprising contacting a compound inducing APP activation with a neuronal cell expressing an APP that induces the death of the cell when activated;

(10) the method of (9), wherein the neuronal cell is the neuronal cell of any one of (1) to (4);

(11) the method of (9), wherein the neuronal cell is a primary cultured cell derived from a brain cortex neuron;

(12) the method of any one of (9) to (11), wherein the compound is an antibody that binds to an APP;

(13) the method of (12), wherein the antibody is an antibody that binds to the extracellular region of an APP;

(14) a method for inducing neuronal cell death associated with APP activation, the method comprising contacting a compound inducing the expression of an active APP mutant with the neuronal cell of any one of (5) to (8);

(15) a method for screening a compound inducing neuronal cell death associated with APP activation, the method comprising:

(a) contacting a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated,

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inducing the death of the neuronal cell;

(16) the method of (15), wherein the neuronal cell is the neuronal cell of any one of (1) to (4);

(17) the method of (15), wherein the neuronal cell is a primary cultured cell derived from brain cortex neuron;

(18) a method for screening a compound inhibiting the induction of neuronal cell death associated with APP activation, the method

comprising:

(a) contacting a compound inducing APP activation and a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated,

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inhibiting the induction of the death of the neuronal cell;

(19) the method of (18), wherein the neuronal cell is the neuronal cell of any one of (1) to (4);

(20) the method of (18), wherein the neuronal cell is a primary cultured cell derived from a brain cortex neuron;

(21) the method of any one of (18) to (20), wherein the compound inducing APP activation is an antibody that binds to an APP;

(22) the method of (21), wherein the antibody is an antibody that binds to the extracellular region of an APP;

(23) a method for screening a compound inhibiting or enhancing the induction of neuronal cell death associated with APP activation, the method comprising:

(a) contacting a compound inducing the expression of an active APP mutant and a test sample with the neuronal cell of any one of (5) to (8),

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inhibiting or enhancing the induction of the death of the neuronal cell;

(24) a kit for screening a compound inducing neuronal cell death or a compound inhibiting or enhancing the induction of neuronal cell death, the kit comprising the neuronal cell of any one of (1) to (8);

(25) an agent for inducing neuronal cell death, the agent comprising as an active ingredient a compound inducing neuronal cell death associated with APP activation;

(26) the agent of (25), wherein the compound is an APP agonist;

(27) the agent of (26), wherein the APP agonist is an antibody that binds to an APP;

(28) the agent of (27), wherein the antibody is an antibody that binds to the extracellular region of an APP;

(29) an inhibitor of neuronal cell death, which comprises as

an active ingredient a compound inhibiting the induction of neuronal cell death associated with APP activation;

(30) the inhibitor of (29), wherein the compound is an APP antagonist;

(31) the inhibitor of (29) or (30), wherein the inhibitor is an agent for preventing or treating a disease caused by neuronal cell death; and

(32) the inhibitor of (31), wherein the disease is Alzheimer's disease.

The present invention provides a method for inducing neuronal cell death associated with APP activation.

An embodiment of the inventive method for inducing cell death is a method comprising contacting a compound inducing APP activation with a neuronal cell expressing an APP that induces the death of the cell when activated. There is no particular limitation on a cell to be used in the method, as long as the cell is a neuronal cell and its death is inducible by the activation of APP being present in the cell. It is preferred to use the cell in which the expression level of an APP has been enhanced by introducing a foreign DNA encoding the APP into the cell for the purpose of efficient induction of cell death by an APP activator. Vectors to be used for APP expression include, for example, pcDNA (C.D. Funk et al., 1990, Proc. Natl. Acad. Sci. USA, 87: 5638-5642), pECE (D.O. Morgan et al., 1987, Nature, 329: 301-307), SRα (T. Takahashi et al., 1996, Lab. Invest., 74: 827-834), and others. The introduction of a vector into cells is achieved, for example, by co-transfecting the vector with the neomycin-resistance gene by utilizing lipofection method. The selected cells are cloned by limiting dilution method. The cloning is conducted by using the expression level of APP as an indicator to provide APP-overexpressing neuronal cell lines. There is no particular limitation on the expression level of APP in cells, as long as cell death is effectively induced by an agent for inducing cell death associated with APP activation. For example, when a neuronal cell line (for example, F11 cell) expressing a foreign APP is used, the expression level is preferably 2 to 100 times as much as that of the endogenous APP, more preferably 5 to 50 times, further preferably 10 to 30 times, yet further

preferably about 20 times. It is possible to appropriately control the expression level of an APP by altering the type of a promoter used or the number of copies of the gene to be introduced.

In the present invention, it is possible to use not only cells expressing the foreign APP but also cells expressing merely endogenous APP, as long as cell death is sufficiently induced by APP activator such as an APP agonist. For example, it is possible to use primary cultured cells derived from brain cortex. In the present invention, cells treated with a differentiation-regulating substances such as retinoic acid are also usable to enhance the expression of endogenous APP.

APP expressed in these cells is a normal APP. In the present invention, "normal APP" means not only the wild-type APP but also APP mutants that are functionally equivalent to the natural one. The term "functionally equivalent to the wild-type APP" herein means having the function of inducing cell death equivalent to that of the wild-type APP.

There is no particular limitation on an APP that is expressed in a cell, as long as the APP is capable of inducing cell death by the treatment of an APP activator. A variety of APPs (members belonging to the APP family, including APLP1 and APLP2) are assumed to be usable for this purpose. Members belonging to the APP family include, for example, mouse APP (K. Yoshioka et al., 1991, Biochem. Biophys. Res. Commun., 178: 1141-1146), human APLP2 (W. Wasco et al., 1993, Nat. Genet., 5: 95-100), mouse YWK-II (Y. C. Yan et al., 1990, Proc. Natl. Acad. Sci. USA, 87: 2405-2408), *Drosophila melanogaster* ALPP (D.R. Rosen et al., 1989, Proc. Natl. Acad. Sci. USA, 86: 2478-2482), mouse APLP1 (S. Zhong et al., 1996, Genomics, 32: 159-162). A preferred example of a protein belonging to the APP family is APP_{695}. APP includes APP mutants as well as the wild-type APP.

In the present invention, there is no particular limitation on the type of APP activator to be used for inducing cell death; for example, an antibody recognizing an APP and, preferably, an antibody recognizing the extracellular region of an APP is usable.

Neuronal cells to be used in the present invention include, for example, cells of nervous system, cells derived from cells of nervous

system, and neural progenitor cells. For example, such cells include neuroblastoma, pheochromocytoma, teratoma, hybrid cells prepared from cells of nervous system, and embryonic germ cell. In the present invention, it is also possible to use neuronal cell lines such as F11 cell, PC12 cell (L.A. Greene and A.S. Tischler, 1976, Proc. Natl. Acad. Sci. USA, 73: 2424-2428), NTERA2 cell (J. Skowronski and M. F. Singer, 1985, Proc. Natl. Acad. Sci. USA, 82: 6050-6054), and SH-SY5Y cell (L. Odelstad et al., 1981, Brain Res., 224: 69-82).

In the present invention, as long as cell death is induced by the treatment of APP activator, the cell is not restricted to neuronal cells expressing human-derived APP but include, for example, neuronal cells expressing any APP (including all members of the APP family) of other organisms such as vertebrates, e.g., other mammals, amphibian, and fish; and invertebrates including *Drosophila* and *Caenorhabditis elegans*.

In the inventive method for inducing cell death, "cell death is induced" means not only that the death event takes place in all the cells but also that the death is significantly induced among the cells. When the cells are treated with an APP activator and the death is induced under optimal conditions, the cell mortality rate is preferably 50% or higher, more preferably 75% or higher, and most preferably 90% or higher. The cell mortality rate can be altered depending on the expression level of APP, the type and amount of APP agonist, duration of the treatment, etc. Those skilled in the art can appropriately select the expression level of APP, and the type and amount of APP agonist, to significantly induce cell death.

In the present invention, it is preferred that cell death is hardly or not induced without the action of any agent capable of inducing cell death associated with APP activation but the death is significantly induced with the action of the inducer. For example, APP_{695} is introduced into F11 cell, which is a hybrid cell between rat primary cultured cell of dorsal root ganglion and mouse neuroblastoma N18TG2 (D. Platika et al., 1985, Proc. Natl. Acad. Sci. USA, 82: 3499; T. Yamatsuji et al., 1996, Science, 272: 1349). Without the action of the monoclonal antibody used as APP agonist, the death is hardly induced in the cell expressing APP_{695} at about 20 times higher level as compared

with the parental F11 cell, but the action of the antibody at a definite or higher concentration results in the induction of the death in almost all the cells.

Another embodiment of the inventive method for inducing cell death is a method comprising contacting a compound inducing the expression of an active APP mutant with a neuronal cell which inducibly expresses the active APP mutant that can induce the death of the cells.

In the present invention, the term "active APP mutant" means an APP of which amino acid sequence has been modified to ligand-independently express APP activity and, specifically, means an APP mutant having the activity of inducing neuronal cell death even in the absence of the ligand. Such APP includes APP mutants of familial Alzheimer's disease (FAD-APP). Known FAD-APP includes APP_{695} mutant having a substitution of Ile, Phe, or Gly for Val at position 642, and another APP_{695} mutant having a substitution of Asn for Lys at position 595 and a substitution of Leu for Met at position 596. Other mutants are also usable in the present invention as long as they have the activity as the active APP mutant. Mutations in the active APP mutants may be spontaneous mutations or artificially introduced mutations. Further, the origin of active APP mutants is not restricted to human; APPs derived from a variety of animals can be used in the present invention.

A neuronal cell inducibly expressing an active APP mutant can be prepared by any of various methods. For example, it is possible to prepare such neuronal cells by introducing a vector containing a DNA encoding an active APP mutant downstream of an inducible promoter into the cells. Usable promoters are, for example, metallothionein promoter, which is induced mainly by heavy metal ions, and tetracycline-inducible promoter, which is induced by tetracycline.

Further, nuclear receptors are thought to be utilized for indirect induction of the active APP mutant expression. In an embodiment of this method, a neuronal cell which contains a vector comprising a DNA encoding a nuclear receptor and a vector comprising a DNA encoding an active APP mutant that is functionally connected downstream of a responsive element for the nuclear receptor is first prepared. The nuclear receptor functions as a transcription factor

by binding to its ligand. When the prepared cell is reacted with the ligand, first, the ligand and the nuclear receptor expressed bind to each other to form a complex, and subsequently the complex binds to the nuclear-receptor responsive element. Then the complex functions as a transcription factor and induces the expression of the DNA encoding the active APP mutant downstream of the responsive element. At the final step, the expression of the active APP mutant induces the death of the neuronal cell expressing active APP mutant.

There is no particular limitation on the type of nuclear receptor used in the method. An example for the receptor is ecdysone receptor (Shuman, S., 1994, J. Biol. Chem. 269: 32678-32684). For example, ecdysone receptor, which is activated by binding with ecdysone, is first expressed together with retinoid X receptor in a cell. Subsequently, the active APP mutant gene is expressed by using cDNA encoding active APP mutant that is inserted downstream of an ecdysone responsive element. Inducible expression of active APP mutant by ecdysone can be achieved by using the obtained cell line. An inducible expression system using Cre in combination with loxP can also be used.

Usable neuronal cells in this method are the same as those in the above-mentioned system of inducing neuronal cell death by the APP activator.

The preparation of vectors for the expression of nuclear receptor and active APP mutant and introduction of the vectors into cell are achieved by using known genetic engineering techniques.

In the inventive vector containing a DNA encoding an active APP mutant functionally connected downstream of a responsive element for the nuclear receptor, "functionally connected" means that a DNA encoding an active APP mutant is connected downstream of a responsive element for the nuclear receptor to secure the expression the mutant.

In this induction system of neuronal cell death using an active APP mutant, "cell death is induced" means not only that the death event takes place in all the cells by the induction of an active APP mutant expression but also that the death is significantly induced among the cells. The cell mortality rate can be altered depending on the type and the expression level of active APP mutant, duration of the expression, etc. These can be appropriately controlled. When

the death is induced by inducing the expression of active APP mutant under optimal conditions, the mortality rate is preferably 50% or higher, more preferably 75% or higher, and most preferably 90% or higher.

The present invention also provides a method for screening an agent inducing cell death associated with APP activation. In this screening method, the cell is contacted with not the APP activator but instead a desired test sample of which activity of inducing cell death associated with APP activation is to be detected; the cell death inducing activity of the test sample is detected; and a compound having the activity is selected by using the above-mentioned cell death induction system using APP activation.

Specifically, the screening method comprises: (a) contacting a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated; (b) detecting the death of the neuronal cell; and (c) selecting a compound inducing the death of the neuronal cell.

The test sample used for the screening includes, for example, purified proteins (including antibodies), expression products from gene libraries, synthetic peptide libraries, cell extracts, cell culture supernatants, libraries of synthetic low-molecular-weight compounds, natural materials such as soil, and solutions containing substances released from bacteria such as culture broth of mycobacterium; but it is not limited to only these examples. Depending on the type of test sample, the contact of test sample with cells can be achieved by adding the test sample to cell culture medium, introducing the test sample into cells (including gene transfer), etc. The detection of cell death can be carried out by the methods as described in Examples. If the result of detection shows significant induction of cell death, the test sample used in the screening can be judged to be a candidate for the agent inducing neuronal cell death.

So far, there is no known naturally occurring APP agonist. The screening of naturally occurring APP agonists can be conducted by using the screening method. For example, it is possible to isolate and identify the gene encoding a protein possibly functioning as an APP agonist or ligand *in vivo*, when an expression library of

brain-derived cDNA is used as a test sample.

Further, when the screening is carried out by using extract from cells of brain as a test sample and the activity of inducing cell death is detected in the sample, the test sample is fractionated and further screened to finally specify as a single compound, which is a constituent of the test sample having the activity of inducing cell death.

In the signal transduction associated with neuronal cell death, there can be many action sites of the compound obtained by the screening. For example, the compound may act directly on APP, or a signal preceding or following APP. The compound that directly acts on APP and induces cell death is particularly called APP agonist in the present invention. APP agonist can be any of substances capable of inducing cell death, as long as the substance can act on cells to activate APP and activated APP induces the death of the cells. For example, an antibody recognizing the extracellular region of APP can be a candidate for APP agonist. As shown in Examples, such antibodies include α1680 antibody (refer to Examples) and Alz90 antibody (Boehringer Mannheim; Code No.1381466), which are monoclonal antibodies recognizing the extracellular region of APP_{695}. There is no particular limitation on the type of antibody. Preferably, monoclonal antibody is used. The monoclonal antibody includes chimeric antibody, humanized antibody, and human antibody. The antibody further includes not only complete antibody molecule but also the Fab fragment, $F(ab')_2$ fragment, single chain Fv (scFv), and the like. These types of antibodies can be prepared according to methods known to those skilled in the art. Antibodies thus prepared are used as APP agonists for inducing cell death. APP agonist can also be a peptide or any other compound. APP agonist may be a naturally occurring or artificially synthesized compound.

Furthermore, the present invention provides a method for screening an inhibitor for neuronal cell death associated with APP activation. In an embodiment of the screening method, the cell is contacted with the APP activator and a desired test sample of which activity of inhibiting the induction of cell death associated with APP activation is to be detected; the activity for the test sample to inhibit the induction of cell death is detected; and a compound

having the inhibitory activity is selected, by using the above-mentioned cell death induction system using APP activation. Specifically, the screening method comprises: (a) contacting a compound inducing APP activation and a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated; (b) detecting the death of the neuronal cell; and (c) selecting a compound inhibiting the induction of the death of the neuronal cell. Common features characteristic of sporadic Alzheimer's disease are as follows: (1) the cell death is associated with APP; (2) the APP is not a mutant; and (3) the cell death takes place in a neuron-specific manner. The neuronal cell death that takes place in this screening system shares all the features, and thus the cell death can be used suitably as a model for neuronal cell death associated with sporadic Alzheimer's disease. Because of this, compounds given by the screening are highly possible to be candidates for drugs for general Alzheimer's disease. The S/N ratio of cell death is extremely high and the procedure used is simple in this system. Accordingly, the system can be utilized as a new high-throughput system for rapid selection of antagonistic agent for Alzheimer's disease.

In a preferred embodiment of the screening method, an APP_{695} cDNA (I, Nishimoto et al., 1993, Nature 362: 75) is inserted into an expression plasmid and then introduced into neuronal cells (for example, F11 cell). The cells into which APP-expression plasmid has been introduced are selected and then an APP activator, such as APP agonist, is allowed to act on the cells. Efficient induction of cell death should be confirmed. Compounds inhibiting the induction of cell death are selected by acting the APP activator on the thus-obtained cells in the presence of a test sample.

The test sample used for the screening includes, for example, purified proteins, expression products from gene libraries, synthetic peptide libraries, cell extracts, cell culture supernatants, libraries of synthetic low-molecular-weight compounds, natural materials such as soil, antisense nucleic acids, and ribozymes; but it is not limited to only these examples.

Depending on the type of the test sample, the contact of the test sample with cells can be achieved by adding the test sample to

cell culture medium, introducing the test sample into cells (including gene transfer), etc. The detection of cell death can be carried out by the methods as described in Examples. If the result of detection shows significant induction of cell death, the test sample used in the screening can be judged to be a candidate for the agent for inhibiting neuronal cell death. The inventive inhibitor includes not only the inhibitor that completely inhibits the cell death but also the inhibitor that significantly lowers the cell mortality rate as compared with that in the absence of the inhibitor.

There is no particular limitation on the action site of the candidate compound for cell death inhibitor selected by this screening. The compound may directly act on APP, or a signal preceding or following APP in the APP-mediated signal pathway for cell death. For example, the compound that acts upstream of APP includes a compound that inhibits the binding of APP agonist or the like, which activates APP, to APP. Further, the candidate compound can be a compound that directly acts on APP and inhibits the binding of APP agonist, or a compound having the activity of preventing the APP activation even when APP agonist binds to APP. The compound can also be a compound blocking cell death signal transduction following APP.

The present invention further relates to a method for screening an inhibitor or enhancer of neuronal cell death associated with APP activation, the method in which the system where cell death is induced by inducibly expressing an active APP mutant. As describe above, active APP mutants were found in familial AD cases, and therefore the cell line can be a suitable model for neuronal cell death in familial AD. The screening method comprises: (a) contacting a compound inducing the expression of an active APP mutant and a test sample with a neuronal cell inducibly expressing the active APP mutant that induces the death of the cell; (b) detecting the death of the neuronal cell; and (c) selecting a compound inhibiting or enhancing the induction of the death of the neuronal cell.

The test sample used for the screening includes, for example, purified proteins, expression products from gene libraries, synthetic peptide libraries, cell extracts, cell culture supernatants, libraries of synthetic low-molecular-weight compounds, natural materials such

as soil, antisense nucleic acids, and ribozymes; but it is not limited to only these examples.

Depending on the type of the test sample, the contact of the test sample with cells can be achieved by adding the test sample to cell culture medium, introducing the test sample into cells (including gene transfer), etc. The detection of cell death can be carried out by the methods as described in Examples. When the result of detection shows significant inhibition of cell death induction, the test sample used in the screening can be judged to be a candidate for the agent for inhibiting neuronal cell death. The inventive inhibitor includes not only the inhibitor that completely inhibits the cell death but also the inhibitor that significantly lowers the cell mortality rate as compared with that in the absence of the inhibitor. Alternatively, when the result of detection shows significant enhancement of cell death induction, the test sample used in the screening can be judged to be a candidate for the agent enhancing neuronal cell death. The inventive enhancer may be an enhancer that significantly increases the cell mortality rate as compared with that in the absence of the enhancer.

It is possible to obtain APP antagonists or compounds blocking the cell death signal following APP sequence by the screening according to the present invention. These compounds can be candidates for drugs for familial AD and are also thought to be used for treating and preventing a variety of diseases including general AD. Further, the screening of the present invention can give compounds enhancing APP-mediated cell death signaling. This screening system can be utilized to test the adverse effects of drugs applied to the nervous system. Specifically, it can be determined whether or not a drug has the adverse effect of worsening neuronal cell death by using the above-mentioned screening system. The system can be used to eliminate drugs having such an adverse effect.

The cell death inhibitor of the present invention is useful as a drug to treat diseases caused by the cell death and such associated with APP. The compound that binds to APP and inhibits APP activation is particularly called APP antagonist in the present invention. Specific inhibition of cell death associated with APP activation can

be achieved by using APP antagonist. Such inhibitors are particularly useful as drugs to treat a variety of diseases caused by APP activation.

There is no particular limitation on the type of disease for which the inhibitor is used, as long as the inhibition of cell death associated with APP or the related-molecules is effective for the treatment of the disease. Such diseases include, for example, Alzheimer's disease. Previous studies have revealed that neuronal cell death takes place in Alzheimer's disease (I. Nishimoto et al., 1997, Adv. Pharmacol., 41: 337-368). The cell death has been suggested to be involved in a certain activation of APP (I. Nishimoto et al., 1998, Neurobiol. Aging., 19: S33-S38). Because of this, the inventive cell death inhibitor is expected to be utilized as a drug to inhibit neuronal cell death in Alzheimer's disease. With the compound, it is also possible to prevent, in addition to Alzheimer's disease, for example, diseases caused by neuronal cell death induced by cerebral ischemia (T. Kirino, 1982, Brain Res., 239: 57-69). Other target diseases, which can be treated or prevented by the inhibitor, are Parkinson's disease with dementia (M. H. Polymeropoulos et al., 1997, Science, 276: 2045-2047), diffuse Lewy bodies (M.G. Spillantini et al., 1998, Proc. Natl. Acad. Sci. USA, 95: 6469-6473), dementia associated with Down's syndrome, and so on. Further, APP-related molecule, APLP1, has been reported to be a causative gene for congenital nephrotic syndrome (Lenkkeri, U. et al., 1998, Hum. Genet. 102: 192-196). Thus, renal diseases such as nephrotic syndrome can also be targets of the treatment and prevention using the inhibitor.

When the inventive cell death inhibitor is a protein or gene, a DNA encoding the protein or a cDNA or the like corresponding to the gene is inserted into an appropriate expression vector. The vector is introduced into a target cell, thereby achieving the inhibition of cell death associated with APP activation. There are many usable vector systems, which are known to those skilled in the art.

The inventive cell death inhibitor can be an antibody. There is no particular limitation on the type of antibody; it includes monoclonal antibody, chimeric antibody, humanized antibody, and human antibody. The antibody further includes not only complete antibody molecule but also the Fab fragment, $F(ab')_2$ fragment, single chain

Fv (scFv), and the like. These types of antibodies can be prepared according to methods known to those skilled in the art. Antibodies thus prepared are useful for the diagnoses of diseases caused by neuronal cell death, treatment with antibody, etc. From the viewpoint of antigenicity, humanized antibody or human antibody is preferably used for the treatment with antibody.

When the inventive cell death inhibitor is a peptide, it may be modified to improve its cell membrane permeability.

When the above-mentioned compounds inhibiting or enhancing neuronal cell death are used as pharmaceuticals, these compounds can be administered to patients directly or after formulated by known pharmaceutical methods. For example, the compound to be administered is appropriately formulated with a pharmaceutically acceptable carrier or mediim, specifically, sterile water or physiological saline, vegetable oil, an emulsifier, a suspending agent, a detergent, a stabilizer, or the like.

Depending on the characteristics of the compound, the administration to a patient can be carried out, for example, percutaneously, intranasally, transbronchially, intramuscularly, intravenously, intrathecally, intraventricularly, or orally. The dose is altered depending on the age, weight, and symptom of each patient, the method of administration, and so on; a suitable dose can be appropriately chosen by those skilled in the art. Further, if the compound can be encoded by a DNA, it is possible to conduct gene therapy using the DNA inserted into a vector for gene therapy. The dose and method of administration are altered depending on the weight, age, symptom of each patient, and such; a suitable dose and an administration method can be appropriately chosen by those skilled in the art.

The present invention further provides a kit for screening a compound inducing neuronal cell death or a compound inhibiting or enhancing the induction. The kit for screening a compound inducing neuronal cell death contains a neuronal cell expressing the above-mentioned foreign APP that can induce the death of the cell when activated.

Further, the kit for screening compounds inhibiting or enhancing

the induction of neuronal cell death contains (i) a neuronal cell expressing the above-mentioned foreign APP that can induce the death of the cell when activated, or (ii) a neuronal cell inducibly expressing the above-mentioned active APP mutant that can induce the death of the cell.

When the kit contains a neuronal cell expressing a foreign APP that can induce the death of the cell when activated, the kit may further contain a compound inducing the activation of the above-mentioned APP.

Further, when the kit contains, as a neuronal cell inducibly expressing an active APP mutant that can induce the death the cell, a neuronal cell which contains a vector comprising a DNA encoding a nuclear receptor and a vector comprising a DNA encoding an active APP mutant that is functionally connected downstream of a responsive element for the nuclear receptor, and which expresses the active APP mutant when induced by contact of a ligand with the nuclear receptor, the kit may further contain a ligand to the nuclear receptor.

Brief Description of the Drawings

Figure 1 shows the expression of APP_{695} in F11, F11/APP, and Bu695 cells analyzed by Western blotting. In this figure, antibodies used are Alz90 antibody (5 μg/ml) in panel 1; α1680 antibody (0.5 μg/ml) in panel 2; and 22C11 antibody (5 μg/ml) in panel 3.

Figure 2 shows a time course of the death of F11/APP cells treated with anti-APP antibody. The closed symbols indicate total cell counts; the open symbols, the number of viable cells. Circle denotes the treatment with α1680 antibody (0.5 μg/ml); square, Alz90 antibody (1 μg/ml); and triangle, nonspecific IgG (1 μg/ml).

Figure 3 shows a time course of the death of F11/APP cells treated with anti-APP antibody. The closed symbols indicate F11 cell; the open symbols, F11/APP cell. Circle denotes the treatment with α1680 antibody (0.5 μg/ml); square, Alz90 antibody (1 μg/ml); diamond, nonspecific IgG (1 μg/ml).

Figure 4 shows the effect of antibody absorption on F11/APP cell death. The death of F11/APP cell was assayed with antigen-absorbed α1680 antibody (absorbed α1680) or mock-absorbed α1680 antibody (mock

absorbed α1680). The values indicated in the histogram were mean values and standard deviations (SD) obtained in 3 to 10 independent experiments. The small inset indicates the results of Western analysis of the absorbed α1680 antibody sample (lane 2) and mock-absorbed α1680 antibody sample (lane 1) treated with HRP-labeled anti-rat IgG antibody. In this inset, the position marked with "heavy chain" corresponds to α1680 antibody.

Figure 5 shows dose response curves of purified anti-APP antibodies in F11/APP cells. F11/APP cells were treated with various concentrations of α1680 antibody (closed square), Alz90 antibody (open square), Jonas antibody (closed circle), or nonspecific IgG (open circle), and the cell mortality rates were measured after 72 hours.

Figure 6 shows schematic illustrations of two possible models for the death induction of F11/APP cell by anti-APP antibody. In the model shown in the left illustration, the anti-APP antibody activates the cell death machinery by acting on cell-surface APP. In the model illustrated at the right, the anti-APP antibody absorbs soluble APP (sAPP) and as a consequence the cell death takes place. In this figure, the mark "⊥" means inhibition or suppression.

Figure 7 shows the effect of culture medium replacement (elimination of sAPP) on the death of F11/APP cell. The media were replaced at 1-hour intervals during 12 hours to test the cell death induction by sAPP elimination. The experimental conditions are indicated schematically above the histogram. In the upper part, "72 hr Alz90" indicates the treatment with Alz90 antibody for 72 hours; "72 hr IgG" indicates the treatment with nonspecific IgG for 72 hours; "12 hr washing" indicates that medium replacement was carried out at 1-hour intervals and thus 12 times in total; "60 hr Ham F-12" means that cells were cultured without antibody but with a medium alone for 60 hours; and "12 hr Alz90" indicates the treatment with Alz90 antibody for 12 hours. The bottom histogram shows the results obtained under the four experimental conditions.

Figure 8 shows the cell death-inducing ability of RMSQ peptide and SRQM peptide. F11/APP cells were treated with lower (10 nM) or higher (100 nM) concentration of each peptide. The cell mortality rate was measured after 72 hours. This figure also contains the results

of negative (IgG; 1 μg/ml IgG) and positive (Alz90; 1 μg/ml Alz90 antibody) controls.

Figure 9 shows experimental results of cell death induction by using insert wells with microfilter. The experimental conditions are schematically indicated in the upper part of the figure. From the left, "IgG + no beads" indicates a negative control experiment using nonspecific IgG but not the beads; "Alz90 antibody + no beads" indicates a positive control using Alz90 antibody but not the beads; "Alz90 antibody + protein G beads" indicates an experiment using an insert well packed with Alz90 antibody-bound protein G beads; "Alz90 antibody + glutathione beads" indicates a control experiment where, instead of protein G beads, glutathione beads were incubated with Alz90 antibody; and "Glutathione beads alone" indicates a control experiment using an insert well packed with glutathione beads alone. The results obtained by the measurements of cell death are shown in the bottom part of this figure.

Figure 10 shows representative micrographs of the cells adhered to the respective wells, which were subjected to the treatment with "IgG + no beads" (IgG), "Alz90 antibody + no beads" (Alz90), "Alz90 antibody + protein G beads" (Alz90 + protein G beads), or "Alz90 antibody + glutathione beads" (Alz90 + glutathione beads) as shown in Figure 9. The cells were observed by using a phase-contrast microscope.

Figure 11 shows TUNEL staining patterns of F11/APP cells, the death of which was induced by the anti-APP antibody. Marked with "IgG" is a pattern of F11/APP cells treated with nonspecific IgG (1 μg/ml); "α1680" is a pattern of F11/APP cells treated with α1680 antibody (0.5 μg/ml). The cells were TUNEL-stained 24 hours after the treatment. "α1680 + DEVD-CHO" indicates the treatment with α1680 antibody (0.5 μg/ml) in the presence of 10 μM Ac-DEVD-CHO; "UV" indicates F11/APP cells treated with UV (320 $\mu J/cm^2$ x 10 seconds). The bright portions correspond to the nuclei where the fragmentation of chromosomal DNA took place.

Figure 12 shows analytical results for DNA laddering. F11/APP cells were treated with Alz90 antibody (1 μg/ml) in the absence (-) or presence (DEVD-CHO) of Ac-DEVD-CHO for 24 hours, and then recovered to detect DNA laddering. The concentrations of Ac-DEVD-CHO used were

0.1, 1, 10, and 100 μM from the left.

Figure 13 shows nuclear morphology of F11/APP cells, the death of which was induced by the anti-APP antibody. F11/APP cells were treated with α1680 antibody and stained by TUNEL method. Nuclear compaction, cleavage, and fragmentation characteristic of apoptosis are observed.

Figure 14 shows the effect of tetrapeptide caspase inhibitors on the death of F11/APP cells induced by anti-APP antibody. F11/APP cells were treated with nonspecific IgG antibody (1 μg/ml), α1680 antibody (0.5 μg/ml), or Alz90 antibody (1 μg/ml) in the absence (-) or presence of each of 10 μM tetrapeptides. The cell mortality was measured after 72 hours.

Figure 15 shows the verification of DNA laddering by the anti-APP antibody in a glioma cell line Bu695 stably overexpressing APP_{695}. Bu695 cells were treated with 1 μg/ml nonspecific IgG or Alz90 antibody (Anti-APP), and the assay for the laddering of DNA contained in the cells was conducted after 24 hours. In an experiment for positive control, Bu695 cells were UV-irradiated (320 $\mu J/cm^2$ x 10 seconds). The sample (UV) after 24 hours was also assayed. In addition, Alz90 antibody-treated F11/APP cells were assayed for a control.

Figure 16 shows TUNEL staining patterns of Bu695 cells. Bu695 cells were treated with nonspecific IgG (1 μg/ml) ("IgG" in the figure), Alz90 antibody (1 μg/ml) ("Alz90" in the figure), or purified α1680 antibody (0.5 μg/ml) ("α1680" in the figure) for 24 hours, and then subjected to TUNEL staining. In the pattern marked with "UV," Bu695 cells were UV-irradiated (320 $\mu J/cm^2$ x 10 seconds) and stained by TUNEL method. Similar experiments were conducted in triplicate and representative results are shown in this figure.

Figure 17 shows the effect of APP-specific antibody on primary cultured cortical neurons. Mouse primary cultured cortical neurons were incubated with 2 μg/ml nonspecific IgG (a, c) or Alz90 antibody (b, d) for 72 hours. Similar but independent experiments were conducted in quadruplicate, and representative results of phase contrast micrographs are shown in a and b. Similarly treated cells were stained with Hoechst 33258, and the results are shown in c and d. Magnifications in a and c are the same as those in b and d, respectively.

Figure 18 shows the TUNEL staining patterns of primary cultured cortical neurons treated with APP specific antibody. The primary cultured cortical neurons were incubated with 2 μg/ml nonspecific IgG or Alz90 antibody in the presence or absence of 10 μM Ac-DEVD-CHO or cell-impermeable Ac-DEVD for 24 hours. Then, DNA fragmentation was analyzed by TUNEL staining. Independent but similar experiments were conducted in triplicate and representative results are shown in this figure.

Figure 19 shows the expression of APP_{695} in FAD-APP-expression inducible cells (V642I). F11/EDXR/V642I cells were cultured in the presence (+) or absence (-) of 20 μM Muristerone (Invitrogen) for 48 hours, and then subjected to immunoblotting using anti-Alz90 antibody (5 μg/ml). The figure shows a typical result of at least 8 experiments.

Figure 20 shows results of incubation of F11/EDXR/V642I cells with (+) or without (-) 20 μM ecdysone in the presence (+) or absence (-) of 10 μM Ac-DEVD-CHO for 48 hours. The cell mortality rate was measured with trypan blue-exclusion assay. The figure shows typical results of at least 8 experiments.

Figure 21 shows micrographs of F11/EDXR/V642I cells cultured in the presence (bottom panel) or absence (top panel) of 20 μM Muristerone for 48 hours. The figure shows typical results of at least 8 experiments.

Figure 22 shows Hoechst-33258 stained patterns of F11/EDXR/V642I cells cultured in the presence (bottom panel) or absence (top panel) of 20 μM Muristerone for 48 hours. The figure shows typical results of at least 8 experiments.

Best Mode for Carrying out the Invention

The present invention is illustrated in detail below with reference to Examples, but is not to be construed as being limited thereto.

Example 1. Anti-APP monoclonal antibody

A monoclonal antibody, α1680 antibody, was prepared as follows. A BglII fragment containing APP1-591 (I. Nishimoto et al., 1993, Nature,

362: 75-79) was inserted into an expression vector containing a GST protein encoding sequence (F. Eckhardt et al., 1997, Mol. Cell. Neurosci., 9: 409-419) and introduced into *E. coli* (DH5α) to produce a fusion protein of GST and APP1-591. The recombinant protein was purified and recovered by using glutathione beads (J.V. Frangioni and B.G. Neel, 1993, Anal. Biochem. 210: 179-187). The monoclonal antibody was prepared by using the fusion protein of GST and APP1-591 as an antigen according to a known method (C.J. Barnstable et al., 1978, Cell, 14: 9-20).

It was confirmed that α1680 antibody did not recognize the recombinant GST although the antibody specifically recognized APP1-591, which covers most of the extracellular region of APP. In the present invention, unless otherwise stated, α1680 antibody solution indicates 10-fold dilution of hybridoma culture medium containing 5 μg/ml α1680 antibody. Alz90 antibody is a mouse monoclonal IgG (Boehringer Mannheim; Code No.1381466) against the amino acid residue 511-608 of the extracellular region of APP.

Example 2. Preparation of neuronal cell expressing APP

The neuronal cell used was F11 cell. F11 cell is a hybrid cell between a neuronal cell derived from first dorsal root ganglion and a neuroblastoma N18TG2, and has characteristics of neuronal cell (D. Platika et al., 1985, Proc. Natl. Acad. Sci. USA, 82: 3499; T.Yamatsuji et al., 1996, Science, 272: 1349). F11 cell was cultured in a Ham F-12 medium (Gibco) containing 18% fetal calf serum. A HindIII-BamHI fragment containing APP_{695} cDNA (I. Nishimoto et al., 1993, Nature, 362: 75) was inserted into an expression vector pcDNA (C.D. Funk et al., 1990, Proc. Natl. Acad. Sci. USA, 87: 5638-5642). Together with pBabe/puro containing the puromycin-resistance gene (H. Zhao et al., 1996, J. Invest. Dermatol. 106: 744-752), the resulting plasmid was introduced into F11 cells by lipofection method. The lipofection was carried out by using APP cDNA (9 μg), pBabe/puro (1 μg), and lipofectamine (10 μl; Gibco) according to a method as described in a reference (T. Okamoto et al., 1996, EMBO J., 15: 3769). Cells were selected by using 14 μg/ml puromycin, and 4 to 5 weeks after the selection, the resistant colonies were recovered. Subcloning was conducted by limiting

dilution method for the colonies exhibiting high-level expression of APP. The expression level was evaluated by immunoblot analysis using 5 μg/ml anti-APP monoclonal antibody 22C11 (T. Okamoto et al., 1995, J. Biol. Chem., 270: 4205).

The isolated APP-introduced F11 cells stably expressing the APP_{695} (F11/APP) were cultured, and then cell homogenates (50 μg protein each) were prepared from F11/APP cells, the parental F11, and Bu695 cells (refer to Example 9). The homogenates were subjected to SDS-PAGE (10% gel), followed by immunoblotting with Alz90 antibody (5 μg/ml; Figure 1, panel 1), α1680 antibody (0.5 μg/ml; panel 2), or 22C11 antibody (5 μg/ml; panel 3) (Figure 1). The results showed that F11/APP cells expressed APP_{695} protein at a high level, and the level is about 20 times as much as that of the parental F11 cells.

Although the three types of anti-APP antibodies used recognize different parts of APP, the three antibodies gave the signal at similar levels in F11/APP cell and Bu695 cell (Figure 1). (A band of 90 to 100 kDa, which was found in F11 cell only by immunoblot analysis with α1680 antibody, was not reproducible.)

Example 3. Induction of death of neuronal cell with introduced APP_{695}

F11/APP cell, into which APP_{695} was introduced, and F11 cell were treated with α1680 antibody or Alz90 antibody. α1680 antibody is a monoclonal antibody against APP1-591, which covers most part of the extracellular domain of APP_{695} protein.

F11/APP cells were plated on a dish in a Ham F-12 medium containing 18% fetal bovine serum. After 24 hours, the cells were washed and then the medium was replaced with a fresh Ham F-12 medium containing nonspecific IgG (1 μg/ml), α1680 antibody (0.5 μg/ml), or Alz90 antibody (1 μg/ml). Then, cell viability was measured by using trypan blue exclusion as the indicator during the time course of incubation. The cells were not washed but pipetted gently to suspend in a medium without serum. 0.1% trypan blue dissolved in PBS was added to the cell suspension at a final concentration of 0.02%, and the resulting mixture was incubated at 37°C for 15 minutes. Then, the viability was measured by using trypan blue exclusion as an indicator (Figure 2). The same experiments as shown in Figure 2 were also performed for F11/APP cell

and F11 cell. Based on the results, the cell mortality rate was represented by (100 - cell viability) (%) (Figure 3).

As shown in Figures 2 and 3, 72 hours after the antibody treatment, the death took place in 80 to 90% of antibody-treated F11/APP cell. Twenty-four hours after the treatment, nearly all the cells were observed to be viable, but 48 hours after the treatment, the mortality increased markedly. A similar experiment was carried out by using F11 cells stably carrying APP_{695}, which were different sets of cells from those used in Figures 2 and 3. The time course and the mortality of cells treated with α1680 antibody were both similar to those in Figures 2 and 3. On the other hand, the death was hardly induced in F11/APP cells treated with nonspecific IgG even 72 hours after the treatment (Figures 2 and 3). In the results of nonspecific IgG treatment shown in Figure 2, the cell count increased after the treatment, and the saturated cell count slightly increased as compared with that prior to the treatment. This results from the freshly changed medium. Further, cell death was hardly induced (Figure 3) even when the parental F11 cells were treated with α1680 antibody. To verify the reproducibility, independent sets of the same experiment as shown in Figures 2 and 3 were repeatedly conducted in triplicate or more; the results obtained were reproducible.

Example 4. Verification of specificity of α1680 antibody to the induction of cell death

The following experiment was conducted, in order to verify that the induction of cell death by α1680 antibody in Example 3 is specific to α1680 antibody. The recombinant fusion protein of GST and APP1-591 produced in *E. coli* was mixed with glutathione-Sepharose, and the resulting mixture was incubated at room temperature for 8 hours to immobilize the fusion protein of GST and APP1-591 on glutathione beads. Each batch of glutathione beads with and without bound APP1-591 (500 µl/tube each) was mixed with a solution of 5 µg/ml α1680 antibody, and the resulting mixture was incubated at room temperature for another 8 hours. The beads were precipitated by centrifugation, and the supernatant was recovered. The antibody solution obtained was diluted 10 times in a similar manner as shown in Example 3, and then used

in the assay for F11/APP cell. Antibody incubated with APP1-591-bound glutathione beads is referred to as "absorbed α1680," and antibody incubated with control glutathione beads is referred to as "mock-absorbed α1680." Ten-fold diluted "absorbed α1680" or "mock-absorbed α1680" was added to a medium containing F11/APP cells. After 72 hours, the cell mortality rate was measured (Figure 4). The values shown in the graph of Figure 4 are mean values and the standard deviations (SD), which were obtained by repeatedly conducting 3 to 10 sets of independent experiments. An aliquot of the supernatant was transferred onto a membrane. After the blocking, the membrane was analyzed by immunoblotting with HRP-labeled anti-rat immunoglobulin antibody (small inset of Figure 4). The signal was detected by using ECL Western Blotting Detection System (Amersham). In the small inset of Figure 4, lane 1 corresponds to "mock-absorbed α1680" and lane 2 corresponds to "absorbed α1680."

As seen in Figure 4, "mock-absorbed α1680" antibody exhibited the cell death-inducing activity at a comparable level to that of unabsorbed α1680 antibody, but "absorbed α1680" antibody hardly induced cell death. The results of immunoblot analysis showed that antibody was efficiently absorbed from the antibody sample by the absorption treatment (lane 2 in the small inset of Figure 4). Based on the above-described results, it can be confirmed that not the buffer to be used for antibody solution or other constituents or contaminants, but α1680 antibody induced the death of F11/APP cell.

Example 5. Examination of the importance of antibody recognizing the APP extracellular region

It was verified that the recognition region of antibody is important in the induction activity of cell death, by using multiple antibodies recognizing different regions of APP protein. The purified anti-APP antibodies used were α1680 antibody, Alz90 antibody, and Jonas antibody (Boehringer Mannheim; Code No. 1584944). Jonas antibody is a mouse monoclonal antibody prepared against the portion of amino acid residue 643-695 within the intracellular region of APP_{695}.

The culture medium of F11/APP cell was replaced by Ham F-12 media containing various concentration of purified anti-APP antibody.

Seventy-two hours after the treatment, cell mortality rate was measured. The result showed that α1680 antibody induced the death of F11/APP cell with highest efficiency and the maximal efficiency was 90% or more (Figure 5). Seventy-two hours after the treatment with 0.5 μg/ml antibody, the death was induced in most of the F11/APP cells. The cell-killing ability of Alz90 antibody was approximately as half as that of α1680 antibody. Seventy-two hours after the treatment with 1 μg/ml Alz90 antibody, the death was induced in about 90% of the cells. The time course profile of cell death induced by Alz90 antibody was essentially the same as that obtained by α1680 antibody (Figure 5). Under the same conditions, death of the parental F11 cells could not be induced by α1680 antibody nor by Alz90 antibody. Further, Jonas antibody, which is an antibody against the portion of amino acid residue 643-695 within the intracellular region of APP_{695}, was tested for the cell death. Contrasted to the results with the antibody against the extracellular region of APP, the results showed that death of F11/APP cell was hardly induced within a concentration range of the antibody up to 1 μg/ml (Figure 5). In addition, the death of the parental F11 cells was not induced by Jonas antibody. These results revealed that the death of APP-expressing cells induced by anti-APP antibody could be ascribed to the action of the antibody on the extracellular region of APP.

Example 6. Examination of the mechanism in the action of anti-APP antibody

<1>

It has been believed that APP is expressed as a membrane-bound protein and then the extracellular region of APP is secreted as a soluble APP (sAPP) out of cell in the normal metabolism. The results of Example 5 raise the high possibility that the anti-APP antibody induces death of F11/APP cell by binding to the extracellular region of APP_{695} protein expressed on the cell surface (Figure 6, the left schematic illustration). In this case, the anti-APP antibody acts on cell-surface APP and then activates the cell death machinery located in the cytoplasm. However, there is another possibility that the soluble APP (sAPP) secreted from F11/APP cell has the function of

preventing cell from the death but absorption of sAPP by the anti-APP antibody induces cell death. In this case, the anti-APP antibody absorbs soluble APP (sAPP) and this results in sAPP inactivation, which induces cell death; and finally the death of F11/APP cell is induced in an antibody-dependent fashion (Figure 6, the right schematic illustration). APP plays an opposite role in each of the above-mentioned two possibilities. APP activates cell death machinery in the left illustration of the same figure, but APP is associated with neuronal cell-protecting system in the right illustration of the same figure.

Concerning the latter possibility, it has been reported that sAPP has neuronal protection effect (J.M. Roch et al., 1994, Proc. Natl. Acad. Sci. USA, 91: 7450; L.W. Jin et al., 1994, Neurosci., 14: 5461). Nonetheless, the possibility shown in the right schematic illustration of Figure 6 is thought to be excluded. One reason for this is as follows: cell viability was not increased in F11 cells overexpressing APP under a cell death-inducing stimulus, such as UV irradiation (the parental F11 cell was subjected to the same experiment as shown in Figure 16, in which F11/APP cell was subjected to UV irradiation; most of the parental F11 cells were killed by apoptosis after 24 hours, which was the same as found with F11/APP cells). Another reason is that the cells overexpressing APP were killed with high frequency by serum deprivation (for example, when treated with nonspecific IgG in the absence of serum, the cell mortality rate is higher in the APP-overexpressing cell than in the parental F11 cell, which is shown in Figure 3).

In order to further examine the above-described latter possibility, the following series of experiments were carried out. <2>

First, the cells were repeatedly washed with media and soluble factors were removed to observe the effect of the removal. The medium of F11/APP cell was replaced by Ham F-12 medium without serum, and the replacement was repeated at 1-hour intervals during the first 12 hours; then the cells were incubated in Ham F-12 medium without serum for 60 hours (Figure 7; 12 hr washing). Assay for cell death was performed 72 hours after the start of the experiment. In other

experiments conduced, F11/APP cells were incubated in Ham F-12 medium containing 1 μg/ml Alz90 antibody for 12 hours (Figure 7; 12 hr Alz90), for 72 hours (Figure 7; 72 hr Alz90), and the cells were incubated in Ham F-12 medium containing 1 μg/ml nonspecific IgG for 72 hours (Figure 7; 72 hr IgG). The experimental conditions are illustrated above the panel in Figure 7.

F11/APP cells were treated with Ham F-12 medium containing Alz90 antibody for the first 12 hours, and then the medium was replaced with Ham F-12 medium without antibody. The culture was continued for further 60 hours. The death was induced in most of F11/APP cells; the efficiency was nearly identical to that obtained when the cells were treated with Alz90 antibody for 72 hours. Accordingly, it was clarified that the treatment with Alz90 antibody for 12 hours was sufficient for the anti-APP antibody to activate the cell death machinery of F11/APP cell. If the cell death induced by the anti-APP antibody were ascribed to the absorption of sAPP from the medium, the experimental results obtained here would indicate that the cell death could be induced by the removal of sAPP for the first 12 hours. Therefore, the medium was replaced with the fresh one at 1-hour intervals during the first 12 hours, that is, the medium replacement was repeated 12 times in total. Subsequently, the cells were cultured in Ham F-12 medium without antibody for 60 hours. By this procedure, every effect of soluble APP (sAPP) can be minimized for the first 12 hours. Seventy-two hours after the start of the experiment, the mortality rate increased very slightly in F11/APP cell as compared with the basal mortality rate induced by the nonspecific IgG (Figure 7); the number of cells killed was negligibly small as compared with that obtained when the cells were treated with the anti-APP antibody for 12 hours.

These results indicate that the death of F11/APP cell induced by the anti-APP antibody does not result from the elimination of soluble APP (sAPP). (The slight elevation of the mortality level with the washing treatment for 12 hours is thought to be ascribed to cell damage caused by repeated medium replacement.)

<3>

In the next step, a peptide, which has been reported to be an

sAPP inhibitor, was tested for its effect. A previous report describes that $R^{330}MSQ^{333}$ peptide, which corresponds to a sequence in the extracellular region of APP_{695}, cancels the neuron-protecting effect of sAPP, when used at a concentration of 10 nM (H. Ninomiya et al., 1993, J. Cell Biol., 121: 879). If the anti-APP antibody inactivated sAPP and thereby induced cell death, the addition of this RMSQ peptide into the medium should induce a similar effect. Then, RMSQ peptide or SRQM peptide was added to the medium at a lower (10 nM) or higher (100 nM) concentration, and the F11/APP cells were incubated for 72 hours. After the incubation, the cell mortality rate was measured (Figure 8). After synthesized, RMSQ peptide and SRQM peptide, in which the original RMSQ sequence was scrambled, were purified to 95% or greater purity. The purified ones were used in the experiment. The first and second columns from the left in Figure 8 correspond to a negative (1 μg/ml nonspecific IgG) and positive (1 μg/ml Alz90 antibody) controls, respectively.

As indicated in Figure 8, neither RMSQ nor SRQM could induce the death of F11/APP cell, when used in a concentration range up to 100 nM. These results also indicate that the cell-killing effect of the anti-APP antibody is not attributable to the blocking of sAPP.

<4>

An experiment in which Alz90 antibody was separated from F11/APP cells but in which the antibody was capable of being in contact with sAPP in medium was conducted, in order to confirm that the anti-APP antibody directly acts on the cell-surface APP. To achieve this purpose, Alz90 antibody (final concentration; 1 μg/ml) was pre-incubated with protein G-bound Sepharose (Pharmacia), and the resultants were packed into an insert well having a micropore filter on the bottom thereof. The insert well was integrated onto a well of 24-well plate containing cultured F11/APP cells on its bottom. A schematic illustration of the experimental outline is shown in the top panel of Figure 9. To secure the sufficient contact of the antibody-bound beads with the soluble APP, the culture plate was being shaken slowly (at 1 rpm) in a CO_2 incubator for the first 24 hours. After 72 hours, there were no beads that happened to come out of the insert well to any wells of the 24-well plate where the cells were cultured.

Seventy-two hours after the start of the incubation, the death of F11/APP cell was hardly induced in a bottom well on which the insert well had been placed (Figures 9 and 10). On the other hand, most of F11/APP cells were killed in a control well of which insert well contained Alz90 antibody alone at the same final concentration but not the beads. In another control experiment, glutathione-Sepharose (Pharmacia), which is incapable of trapping the antibody, was incubated with Alz90 antibody (final concentration 1 μg/ml) and then packed into an insert well, which was used as a control insert well. Most of F11/APP cells were killed when cultured on a bottom well equipped with the control insert well. The mortality rate was similar to that obtained with the insert well containing Alz90 antibody alone but not the beads. Cell death was hardly observed with an insert well containing glutathione-Sepharose or nonspecific IgG (final concentration; 1 μg/ml).

The insert well, which was used in this experiment, has a filter that has 3-μm pores. Therefore, soluble proteins can access to the Alz90 antibody-bound beads. On the other hand, the beads used in this experiment are incapable of passing through the pores, and as a consequence Alz90 antibody bound on the beads cannot act on F11/APP cells under the given conditions. Indeed, Alz90 antibody was not detectable in a culture supernatant incubated with an insert well packed with Alz90 antibody/protein-G Sepharose. On the other hand, after incubated with Alz90 antibody/glutathione-Sepharose in an insert well, the culture supernatant contained almost the same amount of Alz90 antibody as in the supernatant treated with an insert well containing Alz90 antibody alone but not the beads. These results imply that direct interaction of the anti-APP antibody with the cell-surface APP is essential for the induction of cell death.

From these results, together with the results shown in Example 6, it is concluded that each of these anti-APP antibodies functions as an agonistic ligand of APP and directly acts on the cell-surface APP, thereby inducing neuronal cell death.

Example 7. Analysis of death of F11/APP cell

<1>

In order to analyze the characteristics of death of F11/APP cell, which is induced by the anti-APP antibody, DNA fragmentation was detected by TUNEL staining method. Cells to be used in TUNEL assay were placed on a glass slide pre-coated with poly-D-lysine, and pre-cultured in Ham F-12 medium containing 18% fetal bovine serum and an antibiotic. The treatment with antibody was carried out in the absence of serum in the same manner as in Example 6.

F11/APP cells were treated with nonspecific IgG (1 μg/ml) or α1680 antibody (0.5 μg/ml) in a medium for 24 hours. The sample was stained with FITC-labeled dUTP by TUNEL method (Y. Gavrieli et al., 1992, J. Cell Biol., 119: 493-501). F11/APP cells were also treated with α1680 antibody (0.5 μg/ml) in the presence of 10 μM Ac-DEVD-CHO (Peptide Inc.) in the same manner, and then subjected to TUNEL staining. Alternatively, F11/APP cells were treated with ultraviolet-light (UV) irradiation (320 $\mu J/cm^2$ x 10 seconds), followed by TUNEL staining (apoptosis is induced in the cell by UV irradiation). The experiment was repeated at least 3 times, and the representative examples are shown in Figure 11.

When F11/APP cells were treated with nonspecific IgG, TUNEL positive signals were not detectable on the nuclei of the treated cells (Figure 11; IgG). On the other hand, when treated with UV irradiation, DNA fragmentation was detected in almost all the F11/APP cells (Figure 11; UV). When the cells were treated with α1680 antibody, DNA fragmentation was detected in the nuclei of 80 to 90% cells 24 hours after the treatment (Figure 11; α1680). Further, Ac-DEVD-CHO, which is a cell-permeable inhibitor of intracellular caspases, inhibited the α1680 antibody-induced DNA fragmentation (Figure 11; α1680 + DEVD-CHO) (caspase has been reported to be associated with the progress of cell death classified as apoptosis (E. S. Alnemri, 1997, J. Cell. Biochem., 64: 33-42)). This suggests that, in F11/APP cell, the anti-APP antibody activates a genetic program for cell death classified as apoptosis, in which caspases are involved and chromatin fragmentation takes place.

<2>

Further, F11/APP cells were incubated with α1680 antibody in a medium for 24 hours in the same manner. DNA laddering in F11/APP cell was investigated in the presence or absence of Ac-DEVD-CHO. DNA ladder of 180-bp intervals is a basic characteristic of apoptosis, and detection of such DNA ladder in an extracted DNA is important to determine whether or not the death is apoptotic (A.H. Wyllie, 1980, Nature, 284: 555). DNA laddering assay was carried out by using an ApopLadder Ex kit (Takara) according to the attached protocol.

F11/APP cells were incubated with Alz90 antibody (1 μg/ml) in the absence (Figure 12; (-)) or presence of various concentrations of Ac-DEVD-CHO in a medium for 24 hours. DNA laddering was assayed. The Ac-DEVD-CHO concentrations used are 0.1, 1, 10, and 100 μM from the left in the figure.

As indicated in Figure 12, 180-bp DNA ladder was found when F11/APP cells were treated with α1680 antibody. Generation of the ladder is inhibited by Ac-DEVD-CHO. As the concentration of Ac-DEVD-CHO increased, the DNA laddering was strongly inhibited. These data showed that DNA fragmentation induced by the anti-APP antibody is an apoptotic event.

<3>

Further, the nuclear morphology of F11/APP cells treated with α1680 antibody was observed by using TUNEL staining. Twenty-four to 36 hours after the treatment, nuclear morphology changes such as compaction, cleavage, and fragmentation took place in almost all the cells. All the phenomena are the features characteristic of apoptosis. Figure 13 shows the nuclei of α1680 antibody-treated F11/APP cells stained by TUNEL method. The figure displays various features, namely nuclear compaction, cleavage, fragmentation, and such, which are typically found in the nuclei of cells in which apoptosis took place. Whereas the accurate timing of the morphological changes was different from experiment to experiment, the changes were generally induced 24 to 36 hours after the treatment with the anti-APP antibody.

Example 8. Effect of caspase inhibitor

In the next experiment, F11/APP cells were incubated with 1 μg/ml

nonspecific IgG or Alz90 antibody in the presence or absence of 10 μM Ac-DEVD-CHO or Ac-DEVD (without aldehyde modification at the C terminus) (DEVD-AMC, used in *in vitro* caspase substrate assay; Peptide Inc.) in a medium for 72 hours. The treated cells were stained with trypan blue to evaluate cell death. Unlike Ac-DEVD-CHO, Ac-DEVD is incapable of permeating through cell membrane and therefore does not act intracellularly. Thus Ac-DEVD does not inhibit caspase and as a result the compound does not suppress apoptosis. The treatment with 10 μM Ac-DEVD-CHO or Ac-DEVD alone was conducted as a negative control. The results are shown in Table 1. The values indicated are mean values ±SD in at least 5 sets of experiments. In this table, the asterisk "*" means that the cell death induced by Alz90 antibody is inhibited at a significant level of 1% in Student's t test and the mark "§" means that the cell death is not inhibited at a significant level of 1% in Student's t test.

Table 1

Mortality rate of F11/APP cell (%)

The death of F11/APP cell induced by Alz90 antibody was markedly inhibited by 10 μM Ac-DEVD-CHO (Table 1). From these results, it was verified that Ac-DEVD-CHO inhibited apoptosis of F11/APP cell induced by Alz90 antibody, like in the case with α1680 antibody. On the other hand, no inhibition effect on the cell death induced by the antibody was observed with Ac-DEVD, which is unmodified with aldehyde at the C terminus and is cell-impermeable (Table 1). These results obtained

shows that the inhibiting effect of DEVD peptide is achieved by the interaction with an intracellular target.

Another experiment was designed to evaluate whether or not other anti-caspase tetrapeptides are also capable of inhibiting the cell death induced by the anti-APP antibody, and if they can do it, to estimate the degrees of inhibition relative to Ac-DEVD-CHO. The following tetrapeptides are known to be specific inhibitors to caspases.

Ac-YVAD-CHO (Peptide Inc.): inhibitor of the caspase 1 family

Ac-VEID-CHO (Peptide Inc.): inhibitor of caspase 6

Z-IETD-CHO (MBL): inhibitor of caspases 6 and 8

As compared with the inhibitors listed above, Ac-DEVD-CHO is capable of inhibiting nearly all types of caspases, and has the highest affinity for the caspase 3 family in particular. The present inventors treated F11/APP cells with nonspecific IgG, α1680 antibody (0.5 μg/ml), or Alz90 antibody (1 μg/ml) in the presence of each tetrapeptide (10 μM) in a medium, and stained the cells with trypan blue to assay the cell mortality (Figure 14). All the tetrapeptides had an aldehyde group at their end. The results showed that each peptide inhibited cell death induced by the anti-APP antibody, and the degree of inhibition was DEVD>YVAD>VEID>IETD in this order (Figure 14).

Among known caspases, no single caspase is known to be inhibited by the tetrapeptides in the same inhibition profile as shown above. These data suggest that at least two types of caspases or an unidentified caspase should participate in the cell death induced by the antibodies used here. These results are compatible to the finding that the anti-APP antibody triggers apoptosis, which is shown in Example 7. The results also suggest that the blocking property of the tetrapeptides for the death of F11/APP cell induced by α1680 antibody is the same as that of the tetrapeptides for the cell death induced by Alz90 antibody. These data are evidence showing that both antibodies induce cell death mediated by the same intracellular machinery triggered by APP as a common target antigen.

Example 9. Tissue specificity of cell death induced by APP

The cell-killing effect of the anti-APP antibody was studied

by using other types of cells, in order to clarify the tissue-specificity of cell death induced by the anti-APP antibody. In this experiment with different types of cells, the cells used were selected to express APP_{695} at similar levels to that of F11/APP cell. As can be deduced from the fact that the level of endogenous APP is insufficient to induce cell death in F11 cell, the expression level of APP is one of major intracellular determinants of cell-killing effect of the anti-APP antibody. Thus, the present inventors used Bu695 cell (Y. Hayashi et al. 1992, Biochem. Biophys. Res. Commun., 187: 1249), which is a clone of glioma cell line Bu-17 and stably expresses APP_{695}. Immunoblot analysis showed that Bu695 cell expressed APP_{695} at a level comparable to F11/APP cell (Figure 1).

In the same manner as in Example 8, the cells were treated with 1 μg/ml nonspecific IgG or Alz90 antibody in the presence or the absence of 10 μM Ac-DEVD-CHO or Ac-DEVD (without aldehyde modification at the C terminus) for 72 hours. The treated cells were stained with trypan blue to evaluate cell death. The results are shown in Table 2. In this experiment, the treatment with 10 μM Ac-DEVD-CHO or Ac-DEVD alone was conducted as a negative control. The values indicated are mean values±SD in at least 5 sets of experiments.

Table 2

Mortality rate of Bu695 cell (%)

The results showed that, relative to that by the treatment with nonspecific IgG, the mortality rate of Bu695 cell could not be increased

by the treatment with Alz90 antibody even at a high concentration at which the antibody induces death of F11/APP cell with high efficiency (Table 2).

Further, to evaluate whether or not the treatment with the anti-APP antibody results in DNA fragmentation in Bu695 cell, Bu695 cells were treated with 1 μg/ml nonspecific IgG or Alz90 antibody for 24 hours. In a positive control, Bu695 cells were UV-irradiated (320 $\mu J/cm^2$ x 10 seconds) and were recovered as a sample after 24 hours. Alz90 antibody-treated F11/APP cell was used as a control. The results are shown in Figure 15. In addition, Bu695 cells were treated with nonspecific IgG (1 μg/ml), Alz90 antibody (1 μg/ml), or purified α1680 antibody (0.5 μg/ml) for 24 hours. The samples were stained by TUNEL method. As a control, UV-irradiated (320 $\mu J/cm^2$ x 10 seconds) Bu695 cells were stained with TUNEL. These results are shown in Figure 16.

As seen in Figures 15 and 16, DNA ladder observed in UV-irradiated Bu695 cells had band sizes corresponding to those of oligonucleosomes, and TUNEL-positive cells increased markedly, while no DNA ladder was observed in Alz90 antibody-treated Bu695 cells and TUNEL-positive nucleus was hardly found. Similar results were obtained with α1680 antibody, namely the antibody did not induce the death of Bu695 cell (relative to that obtained with nonspecific IgG, the mortality rates of Bu695 cell, the death of which was induced by α1680 antibody in the absence and presence of Ac-DEVD-CHO were 119±4% and 104±2%, respectively). Substantially, DNA fragmentation was not detectable in α1680 antibody-treated Bu695 cell by TUNEL assay (Figure 16).

These data suggest that Bu695 cell is equipped with the apoptotic machinery including DNase but the anti-APP antibody does not induce the death. Hence, it is considered that the intracellular program of apoptosis triggered by APP can work in cells derived from neuron but not from glia.

Example 10. Induction of death of primary cultured neuronal cells

In order to clarify whether or not the death as observed in F11/APP cell can actually take place in the neurons of brain, an experiment was conduced by using primary cultured cells of mouse cortical neuron.

Primary cultured cells of cortical neuron were prepared according

to a method in a reference (Y. Z. Eksioglu et al., Brain Res., 644, 282, 1994). Prepared neuronal cells were incubated with 2 μg/ml nonspecific IgG or Alz90 antibody for 72 hours. Then, the cells were stained with Hoechst 33258 (final concentration; 5 μM) for 1 minute (Figure 17). The cells were TUNEL-stained by using a kit (Boehringer Mannheim) according to the attached protocol (Figure 18).

The primary cultured cells of cortical neuron were treated with Alz90 antibody. Then it was revealed that Alz90 antibody induced death of primary cultured neuronal cell with morphological changes of the nucleus specific to apoptosis. The cell death was not induced by nonspecific IgG used as a control (Figure 17). The enhancement of DNA fragmentation was also recognized in the cell death induced by Alz90 (Figure 17).

The effect of caspase on the cell death was investigated, and the results showed that the cell death was significantly inhibited by Ac-DEVD-CHO, which is a cell-permeable caspase inhibitor, but not by Ac-DEVD (DEVD-AMC; Peptide Inc., used for *in vitro* caspase substrate assay), which has no aldehyde modification at its C terminus nor cell permeability (Figure 18). These results show that neuronal APP functions on the apoptosis-inducing pathway.

Because the death is induced by APP in the primary cultured neuronal cell as well as F11/APP cell, the utility of F11/APP cell is certified as a model for neuronal cell death in the living body. Furthermore, the death associated with APP activation can be induced in cells without foreign APP, suggesting that cell death associated with APP activation can actually take place in the living body.

The present invention has revealed that cell death induced by the anti-APP antibody takes place mediated by an apoptosis-inducing program in neuronal cell. A similar phenomenon has been known for immune cells. The cell-surface Fas/CD95 induces cell death responding to a specific antibody (S. Nagata, 1997, Cell 88: 355). There are three main features in this system; firstly, the cell death induced by the antibody is classified as apoptosis; secondly, the antibody acts on a cell-surface transmembrane protein; and thirdly, cell death is induced by the antibody in tissue-specific manner. The inventive antibody/APP system is also equipped with all the three features,

and transmembrane APP can be assumed to play a similar role in neuronal cell to that of the cell-surface Fas/CD95 in immune cell. There are no homologous regions shared by APP_{695} and Fas/CD95 within their cytoplasmic regions. This raises the possibility that the two utilize distinct intracellular machineries for the start of apoptosis induction pathway. Indeed, it has been reported that the apoptosis associated with the FAD-type mutant of APP is mediated by $G_{\beta\gamma}$ of heterotrimeric G protein, but this is not the case for Fas/CD95 (U. Giambarella et al., 1997, EMBO J. 16: 4897). The expression of Fas/CD95 is not detectable in CNS without any disorder or inflammation (T. Matsuyama et al., 1994, Brain Res. 657: 342; T. Matsuyama et al., 1995, Brain Res. Mol. Brain. Res. 34: 166; G.I. Botchkina et al., 1997, Mol. Med. 3: 765). This suggests that transmembrane APP is expected to generally function for cell death in neuronal apoptosis excluding those associated with disorders and inflammations. Such neuronal apoptosis includes programmed cell death in developing CNS, etc.

Example 11. Construction of inducible expression system for familial AD-type APP mutant (FAD-APP)

Previously, the present inventors demonstrated by using TUNEL staining that transient expression of three types of V642 mutant of APP_{695} (FAD-APPs), which were found in familial Alzheimer's disease (FAD), in F11 cells caused DNA fragmentation (T. Yamatsuji et al., 1996, Science 272: 1349-1352). However, the rate of gene introduction was low and the expression was hardly synchronized in such experiments of transient expression by transfection. These problems make it difficult to conduct detailed analyses for cell death, including whether or not the cell death is apoptotic. The previous system is immature to use for the screening of cell death inhibitors and such.

Then, an attempt was made to construct an inducible FAD-APP expression system where the expression of active APP mutant could be induced by an external stimulus. The expression of FAD-APP (V642I APP) is induced in response to ecdysone in F11 cell line in the constructed system (F11/EDXR/V642I) (Figure 19). First, a vector (pVgRXR; Ecdysone-inducible Mammalian Expression Kit, Invitrogen)

capable of expressing both ecdysone receptor and RXR was transfected into F11 cell, and the transformed cells were selected by Zeocin to obtain F11 cells (F11/EDXR) expressing ecdysone receptor and RXR. In the next step, a full-length V642I APP cDNA (T. Yamatsuji et al., 1996, Science 272: 1349-1352) was inserted into pIND vector (Invitrogen) containing an ecdysone receptor responsive element. The resulting plasmid was introduced into F11/EDXR cells by transfection. The cells were selected by G418. The cells obtained were subjected to limiting dilution and finally F11/EDXR/V642I cell was cloned.

The cells were cultured in Ham F-12 medium containing 10% FBS for 24 hours and then incubated in Ham F-12 medium containing 10% FBS and ecdysone (20 μM; Muristerone). The incubation for 2 to 3 days successfully resulted in the induction of cell death for 80 to 90% of the cells (Figure 20). In a control without ecdysone, cell death was hardly induced. Further, the cell death induced by the addition of ecdysone was inhibited by Ac-DEVD-CHO. Associated with cell death, typical nuclear cleavage, cellular blebbing, and cytoplasmic shrinkage were observed, and the cells were ultimately released from the dish (Figures 21 and 22). Instead of 20 μM Muristerone, 40 μM Ponasterone was used as ecdysone, and the result obtained was the same as the former.

As described above, an induction system was constructed for the expression of familial Alzheimer's disease-type mutant APP or active APP mutant. The induction system has made it possible to analyze the function of familial AD-type APP mutant and to screen inhibitors for cell death associated with FAD-APP. Moreover, the above-mentioned data have demonstrated that APP V642I mutant induces apoptosis and APP mutations found in FAD activate the induction function for apoptosis associated with APP.

Industrial Applicability

The present invention has revealed that APP agonists are capable of inducing death of neuronal cell with very high efficiency. The inventive cell lines have made it possible to screen agents for inducing cell death associated with APP activation, such as APP ligands. An induction system for the expression of active APP mutant is further

provided. These systems have also made it possible to screen agents for inhibiting cell death associated with APP activation, such as APP antagonists. The agents for inhibiting cell death that are obtained by the screening are expected to be used as agents for treating diseases associated with neuronal cell death, including Alzheimer's disease.

CLAIMS

1. A neuronal cell expressing a foreign APP that induces the death of the cell when activated.

2. The neuronal cell of claim 1, wherein the APP is APP_{695}.

3. The neuronal cell of claim 1 or 2, wherein the cell is an F11 cell.

4. The neuronal cell of claim 3, wherein the total intracellular expression level of endogenous APP and the foreign APP is about 20 times as much as the expression level of endogenous APP alone.

5. A neuronal cell inducibly expressing an active APP mutant that induces the death of the cell.

6. The neuronal cell of claim 5, wherein the cell contains a vector comprising a DNA encoding a nuclear receptor and a vector comprising a DNA encoding an active APP mutant that is functionally connected downstream of a responsive element for the nuclear receptor and wherein the expression of the active APP mutant can be induced by contact of a ligand with the nuclear receptor.

7. The neuronal cell of claim 6, wherein the nuclear receptor is an ecdysone receptor and wherein the expression of the active APP mutant is induced by the treatment with ecdysone.

8. The neuronal cell of any one of claims 5 to 7, wherein the cell is an F11 cell.

9. A method for inducing neuronal cell death associated with APP activation, the method comprising contacting a compound inducing APP activation with a neuronal cell expressing an APP that induces the death of the cell when activated.

10. The method of claim 9, wherein the neuronal cell is the neuronal cell of any one of claims 1 to 4.

11. The method of claim 9, wherein the neuronal cell is a primary cultured cell derived from a brain cortex neuron.

12. The method of any one of claims 9 to 11, wherein the compound is an antibody that binds to APP.

13. The method of claim 12, wherein the antibody is an antibody that binds to the extracellular region of an APP.

14. A method for inducing neuronal cell death associated with

APP activation, the method comprising contacting a compound inducing the expression of an active APP mutant with the neuronal cell of any one of claims 5 to 8.

15. A method for screening a compound inducing neuronal cell death associated with APP activation, the method comprising:

(a) contacting a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated,

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inducing the death of the neuronal cell.

16. The method of claim 15, wherein the neuronal cell is the neuronal cell of any one of claims 1 to 4.

17. The method of claim 15, wherein the neuronal cell is a primary cultured cell derived from brain cortex neuron.

18. A method for screening a compound inhibiting the induction of neuronal cell death associated with APP activation, the method comprising:

(a) contacting a compound inducing APP activation and a test sample with a neuronal cell expressing an APP that induces the death of the cell when activated,

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inhibiting the induction of the death of the neuronal cell.

19. The method of claim 18, wherein the neuronal cell is the neuronal cell of any one of claims 1 to 4.

20. The method of claim 18, wherein the neuronal cell is a primary cultured cell derived from a brain cortex neuron.

21. The method of any one of claims 18 to 20, wherein the compound inducing APP activation is an antibody that binds to an APP.

22. The method of claim 21, wherein the antibody is an antibody that binds to the extracellular region of an APP.

23. A method for screening a compound inhibiting or enhancing the induction of neuronal cell death associated with APP activation, the method comprising:

(a) contacting a compound inducing the expression of an active APP mutant and a test sample with the neuronal cell of any one of claims 5 to 8,

(b) detecting the death of the neuronal cell, and

(c) selecting a compound inhibiting or enhancing the induction of the death of the neuronal cell.

24. A kit for screening a compound inducing neuronal cell death or a compound inhibiting or enhancing the induction of neuronal cell death, the kit comprising the neuronal cell of any one of claims 1 to 8.

25. An agent for inducing neuronal cell death, the agent comprising as an active ingredient a compound inducing neuronal cell death associated with APP activation.

26. The agent of claim 25, wherein the compound is an APP agonist.

27. The agent of claim 26, wherein the APP agonist is an antibody that binds to an APP.

28. The agent of claim 27, wherein the antibody is an antibody that binds to the extracellular region of an APP.

29. An inhibitor of neuronal cell death, which comprises as an active ingredient a compound inhibiting the induction of neuronal cell death associated with APP activation.

30. The inhibitor of claim 29, wherein the compound is an APP antagonist.

31. The inhibitor of claim 29 or 30, wherein the inhibitor is an agent for preventing or treating a disease caused by neuronal cell death.

32. The inhibitor of claim 31, wherein the disease is Alzheimer's disease.

ABSTRACT

By preparing neuronal cell stably overexpressing APP and by acting an APP agonist on the neuronal cell, the present inventors developed a system to induce cell death with high efficiency. Preparing a neuronal cell line in which the expression of mutant APP found in familial Alzheimer's disease can be induced by a foreign stimulus, the inventors also succeeded in induction of cell death with high efficiency by inducing the expression of the mutant APP. These systems make it possible to screen agents for controlling cell death in both sporadic and familial Alzheimer's diseases. The agents for inhibiting cell death associated with APP activation that can be obtained by the method of the present invention are useful for the treatment of diseases involved in neuronal cell death including Alzheimer's disease, in which neuronal cell death is necessary to be controlled.